SR 144528, an Antagonist for the Peripheral Cannabinoid Receptor that Behaves as an Inverse Agonist

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ABSTRACT

In the present report, we investigated in detail the effects of SR 144528, a selective antagonist of the peripheral cannabinoid receptor (CB2), on two well-characterized functions mediated by CB2: the induction of the early response gene krox24 and the inhibition of adenylyl cyclase. We generated Chinese hamster ovary cells doubly transfected with human CB2 and a luciferase reporter gene linked to either the murine krox24 regulatory sequence or multiple cAMP responsive elements. Our results show that (1) SR 144528 antagonizes the effect of receptor agonists—it inhibits the krox24 reporter activity and prevents the inhibition of forskolin-induced cAMP reporter ac- tivity mediated by CP 55,940; (2) CB2 is autoactivated—CB2 mediates signaling in the absence of ligand, and this basal activity is reduced by pretreating the cells with pertussis toxin; (3) SR 144528 is an inverse agonist—it reproduces the effects of pertussis toxin; and (4) inhibition of precoupled CB2 by a long-term pretreatment of cells with SR 144528 potentiates krox24 response to cannabinoid receptor agonists and restores activation of adenylyl cyclase. Taken together, these data pro- vide evidences for the inverse agonist property of SR 144528 and the constitutive activation of CB2 in Chinese hamster ovary-expressing cells.

Cannabimimetics have been described as exerting their diverse biological actions through both receptor- and non-receptor-mediated pathways (Lynn and Herkenham, 1994). Cannabinoid receptors belong to the G protein-coupled receptor (GPCR) superfamily and include central (CB1) receptors (Herkenham et al., 1990; Matsuda et al., 1990) and peripheral (CB2) receptors, described predominantly in cells of the immune system (Munroe et al., 1993; Galiègue et al., 1995). Together with the characterization of receptor subtypes and the search for specific antagonists, a considerable effort has been carried out to identify natural endogenous ligands. Such a ligand, arachidonylethanolamide (anandamide), has been isolated from porcine brain and was shown to exhibit the properties of Δ^9-tetrahydrocannabinol (Δ^9-THC), the major psychoactive component of marijuana, in many in vitro and in vivo studies (Devane et al., 1992; Das et al., 1995; Felder et al., 1995). The presence of anandamide was reported in human brain but also in human spleen, which is known to express high CB2 levels (Felder et al., 1996). A second endogenous ligand, 2-arachidonyl glycerol (2-AG), isolated from canine intestines (Mechoulam et al., 1995) and recently iden-

tified in greater amount in rat brain (Stella et al., 1997), was found to modulate biological functions of murine splenocytes (Lee et al., 1995).

Immunoregulatory effects of cannabimimetics have been established for many years (Hollister, 1988). Although the functional role of CB2 remains unclear, its predominant ex- pression in immune tissues taken with in vitro studies on lymphocytes suggests that it specifically mediates both im- munosuppressive and immunostimulatory effects (Derocq et al., 1995; Kaminsky, 1996; Sanchez et al., 1997). The identi- fication of selective ligands (agonists and antagonists) is war- ranted to investigate the respective contribution of CB1 and CB2, and perhaps other cannabinoid receptor subtypes, in functional cannabinoid effects in vivo. Toward this end, we generated Chinese hamster ovary (CHO) cell lines trans- formed with human CB2 and a reporter gene fused to either the regulatory sequences of the early response gene krox24 or multiple copies of cAMP responsive elements (CRE) linked to a minimal promoter. We previously showed that stimulation of cannabinoid receptors was followed by the induction of krox24 protein and activation of its function (Bouaboula et al., 1995a,b, 1996). The physiological relevance of krox24 modulation in the cannabinoid system was also illustrated by

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ABBREVIATIONS: GPCR, G protein-coupled receptor; CB1, central cannabinoid receptor; CB2, peripheral cannabinoid receptor; CHO, Chinese hamster ovary; CKL, CHO cells expressing krox24-luciferase; CKL-CB2, CKL cells expressing CB2; CRE, cAMP responsive element; CCL, CHO cells expressing CRE-luciferase, CCL-CB2, CCL cells expressing CB2; RLU, relative light unit; RLU, relative light unit; PMA, phorbol-12-myristate-13-acetate; AC, adenylyl cyclase; MAPK, mitogen-activated protein kinase; PTX, pertussis toxin; Δ^9-THC, Δ^9-tetrahydrocannabinol; FSK, forskolin.

two independent studies showing a stimulatory effect of Δ^9-THC on krox24 gene expression in rat forebrain and striato-somes (Mailleux et al., 1994; Glass and Dragunow, 1995). Negative coupling of CB1 and CB2 to adenylyl cyclase (AC) is well documented in neuroblastoma or lymphoid cell lines, as well as in nontransformed cells or in heterologous systems (Slipetz et al., 1995; Childers and Deadwyler, 1996; Jung et al., 1997). In immune cells, the modulation of intracellular levels of cAMP by cannabinoid receptor ligands is associated with the regulation of expression of specific genes, including interleukin-2, a cytokine involved in activation of T-cell functions, and inducible nitric oxide synthase (iNOS), which mediates cytolytic effects of macrophages (Kaminsky, 1996).

By using the double transformed CHO cell lines, we investigated the biological responses associated with CB2. Furthermore, we analyzed the effects of the newly described selective CB2 antagonist SR 144528 (Rinaldi-Carmona et al., 1998) on both the cAMP and the krox24 pathways. We show here that SR 144528 not only antagonizes the response elicited by cannabinoid receptor agonists but also acts as an inverse agonist.

Materials and Methods

Reagents. CP 55,940 {(−)-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]4-[3-hydroxypropyl]cyclohexan-1-ol}, SR 144528 {N-[(1S)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]5-(4-chloro-3-methyl-phenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide}, and SR 141716 [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide] were synthesized at the Chemistry Department of Sanofi Recherche (Montpellier, France). WIN 55212-2 {(R)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)meth-yl]pyrrolo[1,2,3-de]1,4-benzoxazin-6-yl](1-naphthyl)methanone} and Ro20−1274 {4-[(3-butoxy-4-methoxyphenyl)methyl]2-imidazolidinone} were from Research Biochemicals Inc. (Natick, MA). Δ^9-THC, anandamide, phorbol-12-myristate-13-acetate (PMA), forskolin (FSK), and pertussis toxin (PTX) were purchased from Sigma Chemical (Saint-Quantin Fallavier, France).

Plasmids. The vector comprising the −395/+65 sequence of the murine krox24 promoter upstream from the coding sequence of firefly luciferase (pUT112-krox24) has already been described (Poinot-Chazel et al., 1996), as has the p661 plasmid containing six consensus cAMP responsive elements (CRE) linked to firefly luciferase (Bouaboula et al., 1997). To generate stable CHO double transformants, human CB2 cDNA was cloned in either pcDNA-3 (InVitrogen, San Diego, CA) or a vector optimized for expression of recombinant proteins in CHO cells (Miloux and Lupker, 1994).

Stable Transfections. Conditions of transfection of CHO cells by electroporation were as previously described (Poinot-Chazel et al., 1996). Cells were first transformed with pUT112-Krox24 (CKL cells) or p661 (CCL cells), before a second round of transformation with the expression vector for CB2, leading to CKL-CB2 cells or CCL-CB2 cells. Cells were screened for CB2 membrane expression and responsiveness of the reporter gene. All cell lines were subcloned by limiting dilution, and one cell clone was selected for each transformation.

Cell Stimulation and Luciferase Assay. Cells were plated onto white 96-well microplates in α-mimimal essential medium (GIBCO BRL, Eragny, France) supplemented with 10% fetal calf serum and incubated for 24 h at 37°C. The next day, the medium was removed and replaced by fetal calf serum-free medium, and cells were further incubated for 24 h at 37°C. For analysis of krox24 or cAMP reporter activities, cells were stimulated for 1.5 and 4 h, respectively. To prepare crude extracts, cells were washed twice with phosphate-buffered saline and lysed using the Cell Culture Lysis Reagent (Promega, Charbonnières, France). Luciferase activities were determined using the Luciferase Assay System (Promega, Madison, WI),

and luminescence was detected using a CCD camera (MTP Reader, Hamamatsu Photonics, Hamamatsu, Japan). Quantification of light emission was obtained by photon counting and mean values from triplicate samples were expressed in relative light units (RLUs). All experiments were repeated at least three times.

cAMP Assays. Measurements of cAMP levels were as previously described (Rinaldi-Carmona et al., 1998). Briefly, cells were incubated for 15 min at 37°C in phosphate-buffered saline supplemented with 0.25% acid-free bovine serum albumin, 0.1 mM isobutylmeth-ylxanthine, and 0.2 mM Ro20-1274 in the presence or absence of 3 × 10^{-9} M CP 55,940. FSK (3 μM) was added, and cells were further incubated for 20 min at 37°C. The reaction was stopped by the addition of ice-cold buffer consisting of 50 mM Tris·HCl, pH 8, and 4 mM EDTA. Extracts were boiled and centrifuged for 10 min at 3500g to remove cell debris. Supernatants were dried, and cAMP concentrations were determined by radioimmunoassay using the scintillant proximity assay system (Amersham, Les Ulis, France).

Western Blot Analysis. Cells were stimulated for 90 min before being lysed in Laemmli's buffer containing 6 M urea. The amount of proteins in each sample was determined by using the Protein Assay kit (BioRad, Ivry sur Seine, France). Preparation of proteins and conditions of Western blotting were as previously published (Poinot-Chazel et al., 1996). Briefly, cell extracts were heated for 10 min at 95°C, and 17 μg of protein per lane was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12% acrylamide gel. Proteins were transferred onto nitrocellulose filters, and blots were hybridized with the anti-krox24 antibody (Santa Cruz, Santa Cruz, CA) at a concentration of 0.25 μg/ml. Immunocomplexes were revealed by a peroxidase-labeled anti-rabbit IgG conjugate associated with the enhanced chemiluminescence detection system (Amersham).

Binding Experiments and Data Analysis. For binding assays, membranes (100 μg) were incubated for 1 h at 30°C with [³H]CP 55,940 in 1 ml of buffer A (50 mM Tris·HCl, pH 7.7). The concentration of [³H]CP 55,940 was 0.2 nM in competition studies and 0.05 to 20 nM in equilibrium binding assays. A rapid filtration technique using Whatman GF/C filters [pretreated with polyethyleneimine 0.5% (w/v) and a 48-well filtration apparatus (Brandel)] was used to harvest and rinse membranes (three times with 5 ml of cold buffer A containing 0.25% bovine serum albumin). The radioactivity bound to the filters was counted with 4 ml of Biofluor scintillant. Nonspecific binding was determined in the presence of 1 μM unlabeled CP 55,940. Data from binding assays were analyzed using a computerized nonlinear least-squares method. All experiments were performed in duplicate, and results were confirmed in at least three independent experiments.

Results

Expression of CB2 in CHO Cell Lines. CHO cells were first transfected with the krox24 reporter (CKL cells) or the cAMP reporter (CCL cells) before a second round of transformation with hCB2 (CKL-CB2 or CCL-CB2 cells), as detailed under *Materials and Methods*. Membrane expression of CB2 was verified by flow cytometry and confocal laser scanning microscopy using the anti-CB2 antibody 4AP (Galiègue et al., 1995) recognizing the C-terminal portion of CB2 (data not shown). We measured the expression level of CB2 in both cell lines by using [³H]CP 55,940 as a ligand. The nonlinear regression analysis of saturation curves revealed the presence of one class of high affinity binding sites for [³H]CP 55,940. The apparent equilibrium dissociation constant (K_D) value and total binding site number (B_{max}) were 0.96 ± 0.24 nM and 2.42 ± 0.38 pmol/mg protein for CKL-CB2 cells and 0.46 ± 0.06 nM and 2.88 ± 0.19 pmol/mg protein for CCL-CB2 cells, respectively. The

specific [^{3}H]CP 55,940 binding was displaced in a concentration-dependent manner by unlabeled CP 55,940, with IC_{50} values of 6.2 ± 0.5 nM for CKL-CB2 cells and 5.3 ± 0.3 nM for CCL-CB2 cells (data not shown).

Effect of Cannabinoid Receptor Agonists on Reporter Activities. The synthetic cannabinoid receptor agonist CP 55,940 induces krox24 reporter activity in CKL-CB2 cells but not CKL cells, with an EC_{50} of 9.3 ± 2.8 nM (Fig. 1A). Western blot analysis confirmed at the protein level that the transgene behaved like the endogenous krox24 gene (Fig. 1B), and quantification of autoradiograms showed a good correlation with results obtained from reporter experiments (data not shown). PMA was used to verify the inducibility of krox24 gene in both the CB2-expressing and nonexpressing cell lines.

On the cAMP reporter, the increase of luciferase elicited by 4-h stimulation with 10^{-6} M FSK was prevented by pretreating CCL-CB2 cells with CP 55,940 (Fig. 1C). The IC_{50} for inhibition by CP 55,940 was 0.29 ± 0.01 nM, indicating a potent functional negative coupling of CB2 to AC. Alternatively, CP 55,940 had no effect on FSK induction of luciferase in parental CCL cells (Fig. 1C). As a control, we also verified the inhibitory effect of CP 55,940 on cAMP levels induced by FSK. Figure 1C shows that 3×10^{-9} M CP 55,940 partially prevented cAMP induced by 3×10^{-6} M FSK, whereas higher concentrations of CP 55,940 led to a complete inhibition of cAMP (data not shown). Similar results were obtained in reporter assays when FSK was used at 3×10^{-6} M instead of 10^{-6} M (data not shown).

In addition to CP 55,940, the effect of the plant cannabinoid Δ^9-THC and the synthetic cannabinoid WIN 55212-2 was investigated. IC_{50} and EC_{50} values from binding and luciferase assays are summarized in Table 1. The biological activities elicited by the drugs were in agreement with their binding potencies, except for Δ^9-THC. In fact, induction of krox24 reporter by Δ^9-THC was extremely low and only detectable with high concentrations ($> 10^{-6}$ M) of the drug. In addition, inhibition of FSK-induced cAMP reporter activity by Δ^9-THC was partial (maximal 60% inhibition), with an IC_{50} of 23.3 ± 2.6 nM. Although this value is similar to its IC_{50} value, Δ^9-THC potency was found more than 1 order of magnitude lower than that of the other compounds investigated. The discrepancy between the CB2 binding efficiency of Δ^9-THC and its weak functional effect in reporter assays led us to investigate whether it could behave as an antagonist for CB2. As shown in Fig. 2, Δ^9-THC shifted the stimulation curve obtained with CP 55,940 to the right. When CP 55,940 was used as 10^{-8} M, the inhibitory effect of Δ^9-THC was detectable at 10^{-8} M and complete at 10^{-6} M. We next examined whether this antagonistic effect could be also obtained with other compounds exhibiting a low affinity for CB2: anandamide, which by itself only slightly stimulated krox24-luciferase in CKL-CB2 cells (1.5–2-fold-increase at 3×10^{-7} M), did not prevent CP 55,940-induced reporter activity (data not shown).

Antagonist Property of SR 144528. We recently described SR 144528 as the first potent and selective CB2 antagonist, which can displace the binding of [^{3}H]CP 55,940 to CHO cells transfected with CB2, with a selectivity for CB2 versus CB1 of 700-fold (Rinaldi-Carmona et al., 1998). Displacement of [^{3}H]CP 55,940 binding by SR 144528 indicated IC_{50} values of 5.85 ± 1.59 nM for CKL-CB2 cells and 6.06 ±
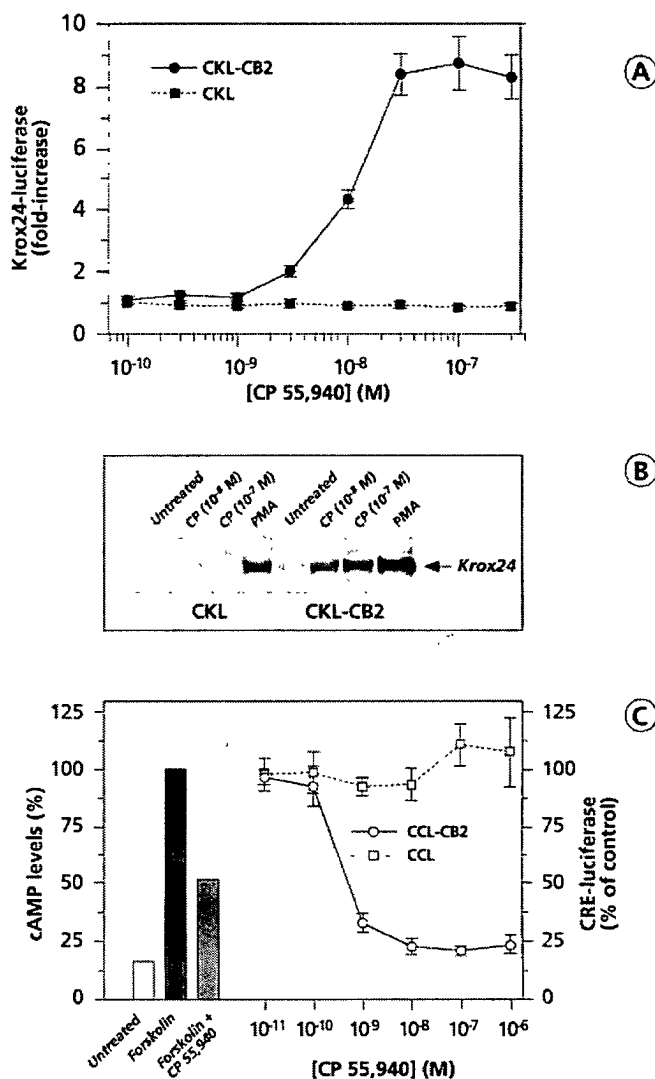

Fig. 1. Effect of cannabinoid receptor agonist on krox24 and cAMP. A, Modulation of krox24 reporter activity. CHO cells expressing krox24-luciferase (CKL, ■) and hCB2 (CKL-CB2, ●) were stimulated for 90 min with increasing concentrations of CP 55,940, as indicated. Cell extracts were prepared and luciferase activities were determined. Data points are the mean of triplicate samples ± S.E.M. Results are expressed as fold-increase, defined by the ratios of RLUs in stimulated versus unstimulated cells. B, Western blot analysis of krox24 expression. CKL cells or CKL-CB2 cells were stimulated for 90 min with 10^{-8} M CP 55,940, 10^{-7} M CP 55,940, or 50 ng/ml PMA, or left untreated. Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose, and hybridized with the anti-krox24 antibody. C, Modulation of cAMP reporter activity. CHO cells expressing the cAMP reporter (CCL, □) and hCB2 (CCL-CB2, ○) were preincubated for 5 min with increasing concentrations of CP 55,940, as indicated, before the addition of 10^{-6} M FSK, and cells were incubated for 4 h before cell extract preparation. Data points are the mean of triplicate samples ± S.E.M. Results are expressed as a percentage of inhibition of FSK-induced luciferase in cells untreated with CP 55,940 (control). Left, modulation of cAMP levels by 3×10^{-6} M FSK, in the presence or absence of 3×10^{-9} M CP 55,940.

1.05 nM for CCL-CB2 cells (data not shown). We next investigated the antagonistic potential of SR 144528 on the modulation of the two reporters by receptor agonists. Figure 3A shows that SR 144528 prevents krox24-driven luciferase induction in CKL-CB2 cells stimulated by CP 55,940. Similar results were obtained when WIN 55212-2 was used as an

TABLE 1

Comparison of cannabinoid receptor ligands for binding and
modulation of reporter activities in CB2-expressing CHO cell lines

Cannabinoid Receptor Ligand	Binding Assay IC_{50}[a]	Krox24-Luciferase Assay EC_{50}[b]	CRE-Luciferase Assay IC_{50}[c]
		nM	
CP 55,940	3.2 ± 0.8	9.3 ± 2.8	0.29 ± 0.01
Win 55212-2	4.9 ± 1.5	11.2 ± 1.6	0.53 ± 0.06
Δ⁹THC	21.4 ± 6.7	>1000	23.3 ± 2.6

[a] Binding data from Shire et al. (1996), obtained using CHO-CB2 cells with a
receptor density similar to that of CKL-CB2 and CCL-CB2 cells.

[b] Luciferase activities from CKL-CB2 cells stimulated with receptor agonists.

[c] Inhibition by receptor agonists of luciferase activities from CCL-CB2 cells stimulated with 10^{-6} M FSK.
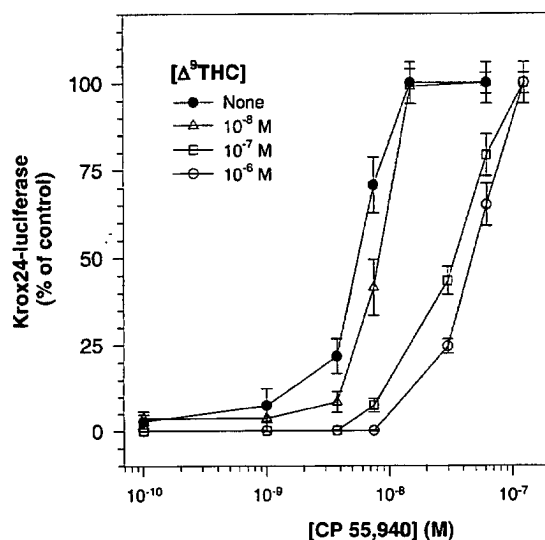
Values are mean ± S.E.M.

Fig. 2. Effect of Δ^9-THC on CP 55,940-induced krox24-luciferase in CKL-CB2 cells. CKL-CB2 cells were pretreated for 5 min with Δ^9-THC (●, control; △, 10^{-8} M; □, 10^{-7} M; ○, 10^{-6} M) before stimulation with CP 55,940 for 90 min, as indicated. Mean luciferase activities from triplicate samples were determined as above and expressed as a percentage of values in cells treated with CP 55,940 alone (control).
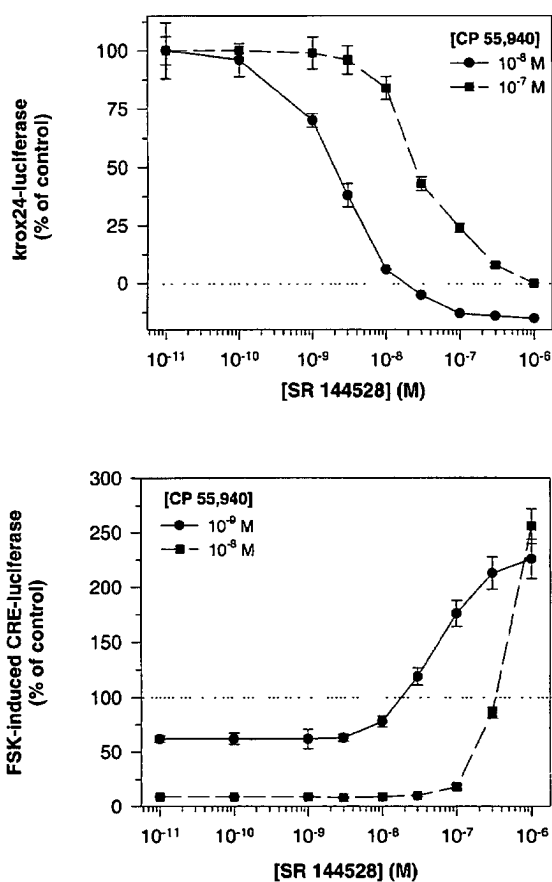

Fig. 3. SR 144528 antagonizes the effect of CP 55,940. A, Inhibition by SR 144528 of CP 55,940-induced krox24-luciferase. CKL-CB2 cells were pre-treated for 5 min with SR 144528, as indicated, before stimulation for 90 min with 10^{-8} M (●) or 10^{-7} M (■) CP 55,940. Mean results are expressed as a percentage of value in cells untreated with SR 144528. B, SR 144528 prevents CP 55,940-mediated inhibition of FSK-induced luciferase. CCL-CB2 cells were preincubated for 5 min with SR 144528 before the addition of medium containing 10^{-6} M FSK and 10^{-9} M (●) or 10^{-8} M (■) CP 55,940. Mean results are expressed as a percentage of value in cells untreated with SR 144528.

agonist (data not shown). Interestingly, when agonist concentration was suboptimal (10^{-8} M), the inhibition of reporter activity by SR 144528 not only reversed the CP 55,940 effect but high concentrations of the antagonist also decreased luciferase values to below baseline level (Fig. 3A). On the other hand, the inhibition mediated by CP 55,940 of FSK-induced cAMP reporter activity was prevented by pretreating CCL-CB2 cells with SR 144528 (Fig. 3B). Again, high concentrations of SR 144528 not only reversed CP 55,940 effect but also led to an increase (2–5-fold) in FSK response above baseline. Under the same conditions, the CB1 antagonist SR 141716 had no effect in both CKL-CB2 or CCL-CB2 cells (data not shown).

Inverse Agonist Property of SR 144528. The latter results showing that SR 144528 effect was not limited to an antagonist property suggested the existence of CB2 pre-coupled to the G protein and suggested that SR 144528 may be an inverse agonist. An enhanced level of signal transduction in the absence of ligand stimulation is associated with receptor autoactivation. Furthermore, treatment of cells with PTX, which selectively ADP-ribosylates G_i/G_o proteins and thus prevents receptor coupling to G protein, is expected to block downstream signaling from such autoactivated recep-

tors. As shown in Fig. 4A, CKL-CB2 cells exhibited a higher constitutive krox24-luciferase activity than the parental cell line, CKL. Similar results were obtained by comparing the basal amounts of endogenous krox24 protein in both cell lines: quantification of the signals on the autoradiogram shown in Fig. 1B indicated a 2.85-fold increase of krox24 protein in CKL-CB2 cells versus CKL cells. Accordingly, treatment of CKL-CB2 cells for 4 h with 50 ng/ml PTX decreased basal activity to the level in parental cells, whereas it did not affect these latter (Fig. 4A). An inverse agonist for a G_o/G_i-coupled receptor, by preventing constitutive activity, is expected to reproduce the effect of PTX. Incubation of CKL-CB2 cells with 10^{-7} M SR 144528 for various lengths of time led to a decrease of basal activity that is consistent with such a property (Fig. 4B). In control experiments, the selective CB1 antagonist SR 141716 had no effect under the same conditions, whereas SR 144528 did not modulate constitutive luciferase activity in CKL cells, indicating that the above results are specifically receptor mediated (data not shown).

Similar results were obtained by investigating the cAMP-regulated pathway. First, pretreatment of CCL-CB2 cells
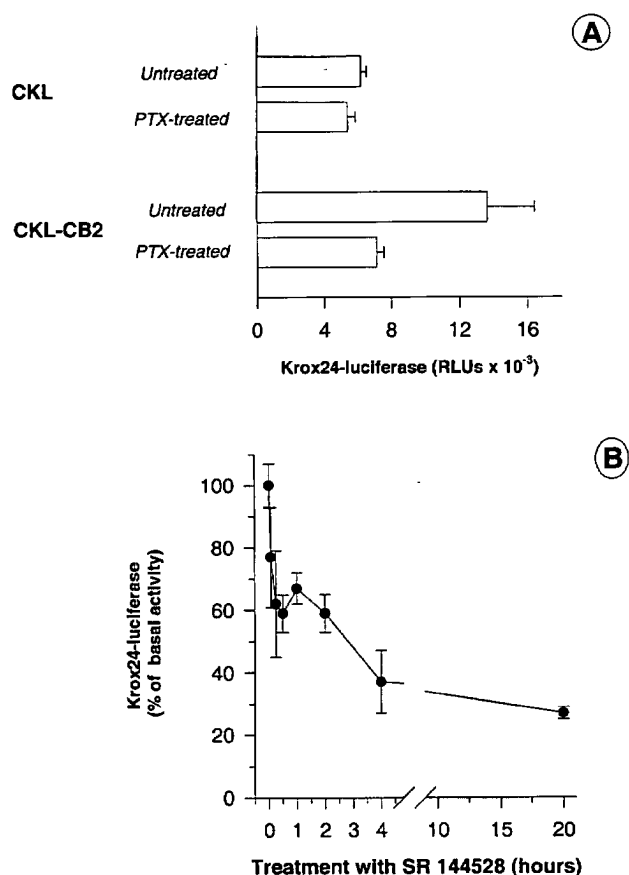
Fig. 4. Effect of PTX and SR 144528 on constitutive krox24 reporter activity. A, CKL-CB2 or CKL cells were treated for 4 h with 50 ng/ml PTX or the diluent (untreated). Cell extracts were prepared and luciferase activities from triplicate samples were determined. Mean results are expressed in RLUs $\pm$ S.E.M. B, CKL-CB2 cells were incubated with 10^{-7} M SR 144528 for the indicated time. Results are expressed as a percentage of luciferase activity in cells treated with the diluent for each time point.

with PTX shifts the FSK stimulation curve to the left (Fig. 5A), without affecting it in CCL cells (data not shown). EC_{50} for FSK in the presence or absence of PTX were 0.43 $\pm$ 0.02 and 4.8 $\pm$ 0.56 μM for CCL-CB2 cells and 0.64 $\pm$ 0.01 and 0.67 $\pm$ 0.01 μM in CCL cells, respectively. Second, SR 144528 potently stimulates CRE-luciferase induced by 10^{-6} M FSK in CCL-CB2 cells, without affecting the FSK response in CCL cells (Fig. 5B). The stimulatory curve is consistent with a CB2-mediated effect because maximal promoting effect was obtained with 3 $\times$ 10^{-8} M SR 144528.

Enhancement of CB2-Mediated Responses by SR 144528. Inverse agonists, in addition to their inhibitory potential of constitutive signaling, were described in a number of case to up-regulate GPCR expression. Thus, we investigated whether sustained treatment of cells with SR 144528 altered agonist-induced signaling. Cells were treated for 18 h with increasing concentrations of SR 144528 or the vehicle, extensively washed, and further stimulated for 90 min with medium containing CP 55,940. As illustrated on Fig. 6A, an increase of the relative level of induction by CP 55,940 of the krox24 reporter was observed. Optimal results were obtained when SR 144528 was used as the concentration of 10^{-8} M, whereas higher concentrations did not further increase the
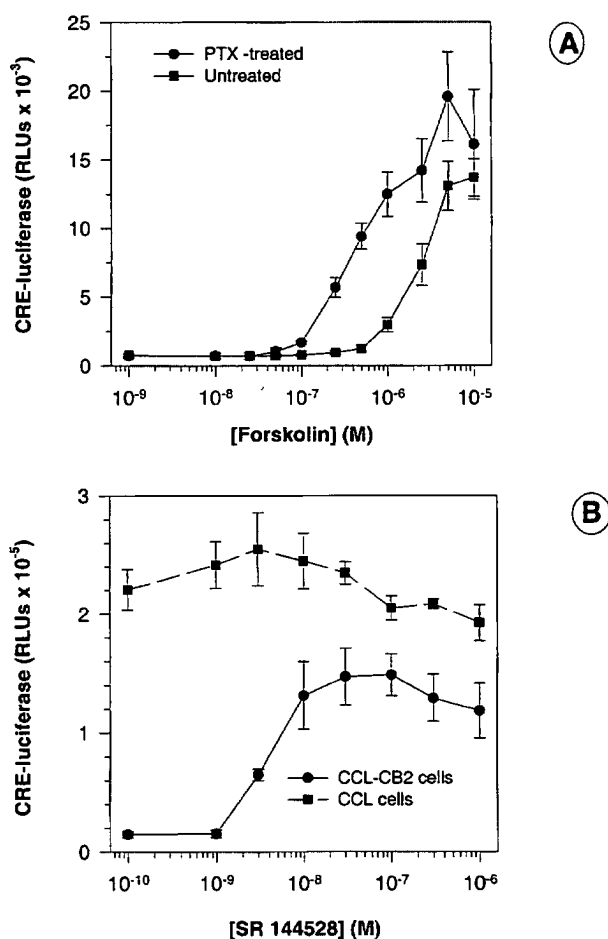
Fig. 5. Effect of PTX and SR 144528 on FSK-induced cAMP reporter activity. A, CCL-CB2 cells were pretreated for 4 h with 50 ng/ml PTX or the diluent before stimulation with increasing concentrations of FSK, as indicated. Cells extracts were prepared after 4 h of stimulation, and mean luciferase activities from triplicate samples were determined. B, CCL (■) or CCL-CB2 (●) cells were pretreated for 5 min with increasing concentrations of SR 144528, as indicated, before the addition of 10^{-6} M FSK. Luciferase activities from triplicate samples were measured after 4 h of incubation. Results are expressed in RLUs $\pm$ S.E.M.

functional response. When examining precisely the effect of SR 144528, we observed that in addition to the expected reduction of luciferase basal level by the inverse agonist, the relative level of induction by CP 55,940 was promoted by ~3-fold. On the other hand, a sustained pretreatment with CP 55,940 (10^{-8} M) or Δ^9-THC (10^{-7} M), followed by an extensive washing, completely prevented further stimulation with CP 55,940 (Fig. 6B). Finaly, the promoting effect of SR 144528 (10^{-8} M) was abolished when CP 55,940 or Δ^9-THC was added during the time of pretreatment (data not shown). Similar experiments performed with CCL-CB2 cells confirmed that prolonging the time of cell pretreatment with SR 144528 further raised the level of FSK-induced CRE-luciferase activity (data not shown).

Discussion

We generated two reporter models consisting of CHO cells doubly transformed with hCB2 and a luciferase reporter gene linked to regulatory sequences of the early response gene krox24 or a CRE to investigate the functional activation of
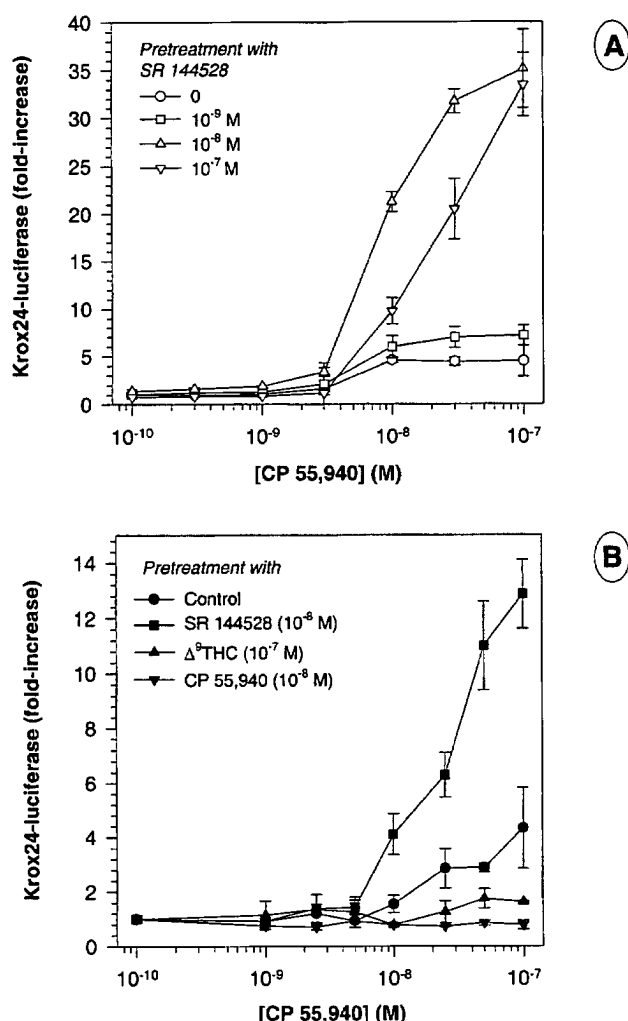
Fig. 6. Effect of a long-term SR 144528 treatment on krox24 response. A, CKL-CB2 cells were pretreated for 18 h with increasing concentrations of SR 144528 or the vehicle, as indicated. The medium was removed, and cells were washed and further stimulated for 90 min with increasing concentrations of CP 55,940. Mean luciferase activities are expressed as fold-increase, defined by the ratios of RLUs in stimulated versus unstimulated cells ± S.E.M. B, CKL-CB2 cells were pretreated for 18 h with either SR 144528, Δ⁹-THC, CP 55,940, or the vehicle, as indicated; extensively washed; and further stimulated with CP 55,940 as above.

CB2 by cannabinoid receptor ligands, including the newly described antagonist SR 144528.

We first studied the pharmacological profile of classic ligands and we observed a similar order of potency when comparing luciferase assays and binding experiments, except for Δ^9-THC, which behaves as an antagonist for the CB2 receptor in the krox24-luciferase assay. This latter result is in agreement with data showing that the inhibition of FSK-induced cAMP levels by the ligands HU-210 and HU-293a was prevented by Δ^9-THC in CHO cells expressing CB2 (Bayewitch et al., 1996). Thus the described effects of Δ^9-THC on immune functions must be carefully reexamined by considering that they may not be the result of a direct stimulation of CB2 but rather an inhibition of the effect of an endogenous ligand. In addition, the antagonist potential of Δ^9-THC for CB2 associated with its agonistic effect for CB1 could explain the contradictory conclusions on immunosuppressive

or immunostimulatory properties of the drug in biological situations where both receptors are expressed.

It is noteworthy that half-maximal values from cAMP reporter assays are more than 1 order of magnitude lower than those from krox24 reporter assays. We have previously shown that the induction of krox24 gene mediated by CB2 paralleled the activation of MAPK in CHO cells expressing CB2 but also in the human promyelomonocytic cell line HL60 (Bouaboula et al., 1996). In additional experiments, we observed that the inhibitor of MAPK kinase PD 098059, as well as the inhibitor of phosphatidylinositol-3-kinase wortmannin, completely prevented induction of krox24 reporter by 10^{-7} M CP 55,940 in CKL-CB2 cells, with IC_{50} of 3.9 ± 0.7 μM and 32.1 ± 4.4 nM, respectively (data not shown). This results favor the hypothesis that the krox24 pathway may be activated through the $G_{\beta\gamma}$ subunits. Because we could observe a correlation between half-maximal values from CRE-luciferase and cAMP assays on the one hand and krox24-luciferase and MAPK assays on the other, the difference observed for cannabinoid response of both reporter systems is rather the result of the use of distinct signaling pathways—one mediated by the *beta gamma* subunits and the other by the *alpha* subunit of the G protein. Our results could account for a more potent coupling of CB2 to the *alpha* subunit or indicate that the signal amplification is more efficient for the cAMP pathway. The potency of the CRE-luciferase system makes it a powerful tool to screen for cannabinoid receptor agonists, whereas antagonists are more directly evidenced through the positively regulated krox24 target.

Functional Properties of SR 144528. We recently characterized SR 144528 as a selective CB2 antagonist (Rinaldi-Carmona et al., 1998). Here, we investigated the effect of SR 144528 on CB2-mediated functional responses by measuring reporter activities. As expected, induction of the krox24 reporter by CP 55,940 is inhibited by SR 144528 in a concentration-dependent manner, whereas SR 144528 prevented the inhibitory effect of CP 55,940 on FSK induction of CRE-luciferase. These results are in line with previous those on MAPK activation and modulation of cAMP levels (Rinaldi-Carmona et al., 1998). Because most cannabinoid receptor agonists are weakly selective or nonselective, SR 144528 thus represents a valuable tool as potent as SR 141716 for evaluating the relative contribution of peripheral and central receptor subtypes in cells coexpressing both receptors or in heterogeneous cell populations.

In the second part of the study, evidence accumulated that CB2 couples to the G protein in the absence of stimulation by receptor agonist and that SR144528 acts as an inverse agonist. The first observations are the constitutive activation of krox24 pathway and the constitutive repression of cAMP pathway, both being prevented by the decoupling agent PTX. Transient transfections of CHO cells with increasing concentrations of CB2 and either krox24 or cAMP reporter plasmids confirmed the direct link between CB2 levels and reporter activities (data not shown), supporting the notion of functional receptors precoupled to the G protein. As expected for an inverse agonist, SR 144528 prevented spontaneous CB2 signaling in overexpressing CHO cells. The inhibition of krox24 gene expression was related to concentration and to the duration of treatment, with a 40% decrease observed after 1 h of treatment with 10^{-7} M SR 144528 and a maximal 75% decrease after 20 h of treatment. Because the half-life of

luciferase (around 3 h) is significantly higher than that of krox24 protein (less than 1 h), this can explain the delayed response in reporter assay, although SR 144528 may act within minutes for decoupling the receptor from the G protein. When investigated on the cAMP pathway, SR 144528 stimulated FSK response, which is consistent with the blocking of the $G_{\alpha i}$-mediated inhibition of AC activity. A correlation between basal cellular activity and GPCR expression was originally described by the identification of activating point mutations, either spontaneous or artificially engineered, or by overexpressing receptors in mammalian or insect cells (Kenakin, 1996; Scheer and Cotecchia, 1997). Increasing receptor levels are associated with the increase in the number of precoupled receptors and, thus, basal activity. Results in agreement with this have been established for *beta* adrenergic receptors (Samama et al., 1993; Chidiac et al., 1994). The potent effect of PTX on basal activity generated by CB2, as well as the relatively low level of induction of the krox24 target by receptor agonists and, on the other hand, the high level of repression of AC, suggests that precoupled receptors are present to a non-negligible extent.

It has been described that GPCR ligands can regulate the level of receptor gene transcription or translation, the turnover of membrane receptors, or the strength of receptor coupling by modulating the pool of G proteins (Kenakin, 1996). For histamine H_2 receptor or *beta*-2 adrenoceptor, a prolonged agonist exposure induces a significant decrease in receptor density, whereas inverse agonists generate the opposite effect (McEwan and Milligan, 1996a; Smit et al., 1996). For the *beta*-2 adrenoceptor, the up-regulation of the constitutively active form by inverse agonist does not occur at the transcriptional level but rather at the translational or post-translational level because it is prevented by cycloheximide (McEwan and Milligan, 1996b). We thus investigated the effect of a long-term exposure to SR 144528, postulating that a modulation of the number of receptor sites per cell would lead to an altered response to further stimulation with receptor agonist. The potent observed effect, a 10-fold higher level of induction by CP 55,940 of krox24 reporter in cells pretreated with SR 144528 versus cells pretreated with the vehicle, account for this to effectively occur. Furthermore, pretreatment with CP 55,940 completely inhibits further functional response, which is consistent with receptor desensitization by the agonist. Thus, our results favor the hypothesis of an up-regulation of the number of CB2 sites by SR 144528 and a down-regulation by CP 55,940. Interestingly, we also observed that pretreatment with Δ^9-THC induced receptor desensitization. This result is in agreement with the expected effect of an agonist, even if it exhibits a low intrinsic activity. On the other hand, we cannot exclude the possibility that receptor desensitization may be attributed to the antagonist potential of Δ^9-THC. Also, there are few examples in the literature; such a property was recently attributed to the antagonist analog of cholecystokinin (Roettger et al., 1997). In addition, the authors demonstrated that receptor internalization occurred independently of the phosphorylation status of the cholecystokinin receptor, establishing that regulation of receptor endocytosis is independent of receptor signaling (Rao et al., 1997). The original property of SR 144528 on the modulation of CB2 functional response in transfected CHO cells may be attributed to its inverse agonist property, and further investigations are required to de-

termine whether SR 144528 has similar effects in cells that naturally express low levels of CB2.

We recently demonstrated that CB1 expressed in CHO cells are also autoactivated and that SR 141716 acts as an inverse agonist in this system (Bouaboula et al., 1997). Similar results using CB1-transfected CHO cells were reported by the group of Yamamura (Landsman et al., 1997). The availability of inverse agonists, for which the list has increased these past few years (Kenakin, 1996), may contribute to a better understanding of receptor behavior in physiological conditions and/or altered conditions such as constitutively activated receptors, although their intrinsic benefit over neutral antagonists in clinical trials is still purely speculative (Milligan et al., 1995). The use of SR 144528 to specifically prevent CB2 activation could be a means to elucidate its function, which remains unclear.

References

Bayewitch M, Rhee MH, Avidor-Reiss T, Breuer A, Mechoulam R and Vogel Z (1996) (−)-Δ^9-Tetrahydrocannabinol antagonizes the peripheral cannabinoid receptor-mediated inhibition of adenylyl cyclase. *J Biol Chem* 271:9902–9905.

Bouaboula M, Bourrié B, Rinaldi-Carmona M, Shire D, Le Fur G and Casellas P (1995a) Stimulation of cannabinoid receptor CB1 induces krox24 expression in human astrocytoma cells. *J Biol Chem* 270:13973–13980.

Bouaboula M, Perrachon S, Milligan L, Canat X, Rinaldi-Carmona M, Portier M, Barth F, Calandra B, Pecceu F, Lupker J, Maffrand JP, Le Fur G and Casellas P (1997) A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1: Evidence for a new model for receptor/ligand interactions. *J Biol Chem* 272:22330–22339.

Bouaboula M, Poinot-Chazel C, Bourrié B, Canat X, Calandra B, Rinaldi-Carmona M, Le Fur G and Casellas P (1995b) Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1. *Biochem J* 312:637–641.

Bouaboula M, Poinot-Chazel C, Marchand J, Canat X, Bourrié B, Rinaldi-Carmona M, Calandra B, Le Fur G and Casellas P (1996) Signalling pathway associated with stimulation of CB2 peripheral cannabinoid receptor: Involvement of both mitogen-activated protein kinase and induction of Krox24 expression. *Eur J Biochem* 237:704–711.

Chidiac P, Hebert TE, Valiquette M, Dennis M and Bouvier M (1994) Inverse agonist activity of β-adrenergic antagonists. *Mol Pharmacol* 45:490–499.

Childers SR and Deadwyler SA (1996) Role of cyclic AMP in the actions of cannabinoid receptors. *Biochem Pharmacol* 52:819–827.

Das SK, Paria BC, Chakraborty I and Dey SK (1995) Cannabinoid ligand-receptor signalling in the mouse uterus. *Proc Natl Acad Sci USA* 92:4332–4336.

Derocq JM, Segui M, Marchand J, Le Fur G and Casellas P (1995) Cannabinoids enhance human B-cell growth at low nanomolar concentrations. *FEBS Lett* 369:177–182.

Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A and Mechoulam R (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science (Washington DC)* 258:1946–1949.

Felder CC, Joyce KE, Briley EM, Mansouri J, Mackie K, Blond O, Lai Y, Ma AL and Mitchell RL (1995) Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. *Mol Pharmacol* 48:443–450.

Felder CC, Nielsen A, Briley EM, Palkovits M, Priller J, Axelrod J, Nguyen DN, Richardson JM, Riggin RM, Koppel GA, Paul SM and Becker GW (1996) Isolation and measurement of the endogenous cannabinoid receptor agonist anandamide in brain and peripheral tissues of human and rat. *FEBS Lett* 396:213–235.

Galiègue S, Mary S, Marchand J, Dussossoy D, Carrière D, Carayon P, Bouaboula M, Shire D, Le Fur G and Casellas P (1995) Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur J Biochem* 232:54–61.

Glass M and Dragunow M (1995) Induction of the Krox 24 transcription factor in striatosomes by a cannabinoid agonist. *Neuroreport* 6:241–244.

Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, de Costa BR and Rice KC (1990) Cannabinoid receptor localization in brain. *Proc Natl Acad Sci USA* 87:1932–1936.

Hollister LE (1988) Marijuana and immunity. *J Psychoactive Drugs* 20:3–8.

Jung M, Calassi R, Rinaldi-Carmona M, Chardenot P, Le Fur G, Soubrié P and Oury-Donat F (1997) Characterization of CB1 receptors on rat neuronal cell cultures: Binding and functional studies using the selective receptor antagonist SR 141716. *J Neurochem* 68:402–409.

Kaminsky NE (1996) Immune regulation by cannabinoid compounds through the inhibition of the cyclic AMP signalling cascade and altered gene expression. *Biochem Pharmacol* **52**:1133–1140.

Kenakin T (1996) The classification of seven transmembrane receptors in recombinant systems. *Pharmacol Rev* **48**:413–463.

Landsman RS, Burkey TH, Consroe P, Roeske WR and Yamara HI (1997) SR 141716A is an inverse agonist at the human cannabinoid CB1 receptor. *Eur J Pharmacol* **334**:R1–R2.

Lee M, Yang KH and Kaminski NE (1995) Effects of putative cannabinoid receptor ligands anandamide and 2-arachidonyl-glycerol on immune function in B6C3F1 mouse splenocytes *J Pharmacol Exp Ther* **275**:529–536.

Lynn AB and Herkenham M (1994) Localization of cannabinoid receptors and non-saturable high density cannabinoid binding sites in peripheral tissues of the rat: Implications for receptor-mediated immune modulation by cannabinoids. *J Pharmacol Exp Ther* **268**:1612–1622.

Mailleux P, Verslype M, Preud'homme X and Vanderhaeghen J (1994) Activation of multiple transcription factor genes by tetrahydrocannabinol in rat forebrain. *Neuroreport* **5**:1265–1268.

Matsuda LA, Lolait SJ, Brownstein MJ, Young AC and Bonner TI (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature (Lond)* **346**:561–564.

McEwan DJ and Milligan G (1996a) Up-regulation of a constitutively active form of the β2-adrenoceptor by sustained treatment with inverse agonists but not antagonists. *FEBS Lett* **399**:108–112.

McEwan DJ and Milligan G (1996b) Inverse agonist-induced up-regulation of the human β₂-adrenoceptor in transfected neuroblastoma X glioma hybrid cells. *Mol Pharmacol* **50**:1479–1486.

Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Shatz A, Gopher AR, Almog S, Martin BR, Compton DR, Pertwee RG, Griffin G, Bayewitch M, Barg J and Vogel Z (1995) Identification of an endogenous 2-monoglyceride present in canine gut that binds to cannabinoid receptors. *Biochem Pharmacol* **50**:83–90.

Milligan G, Bond RA and Lee M (1995) Inverse agonism: Pharmacological curiosity or potential therapeutic strategy? *Trends Pharmacol Sci* **16**:10–13.

Miloux B and Lupker JH (1994) Rapid isolation of highly productive recombinant Chinese hamster ovary cell lines. *Gene* **149**:341–344.

Munro S, Thomas KL and Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature (Lond)* **365**:61–65.

Poinot-Chazel C, Portier M, Bouaboula M, Vita N, Pecceu F, Gully D, Monroe JG, Maffrand JP, Le Fur G and Casellas P (1996) Activation of mitogen-activated protein kinase couples neurotensin receptor stimulation to induction of the primary response gene Krox24. *Biochem J* **320**:145–151.

Rao RV, Roettger BF, Hadac EM and Miller LJ (1997) Roles of cholecystokinin receptor phosphorylation in agonist-stimulated desensitization of pancreatic acinar cells and receptor-bearing hamster ovary cholecystokinin receptor cells. *Mol Pharmacol* **51**:185–192.

Rinaldi-Carmona M, Barth F, Millan J, Derocq JM, Casellas P, Congy C, Oustric D, Sarran M, Bouaboula M, Calandra B, Portier M, Shire D, Brelière JC and Le Fur G (1998) SR 144528, the first potent and selective antagonist of the CB2 cannabinoid receptor. *J Pharmacol Exp Ther* **284**:644–650.

Roettger BF, Ghanekar D, Rao RV, Toledo C, Yingling J, Pinon D and Miller LJ (1997) Antagonist-stimulated internalization of the G protein-coupled cholecystokin receptor. *Mol Pharmacol* **51**:357–362.

Samama P, Cotecchia S, Costa T and Lefkowitz RJ (1993) A mutation-induced activated state of the β-adrenergic receptor: Extending the ternary complex model. *J Biol Chem* **90**:10365–10369.

Sanchez C, Velasco G and Guzman M (1997) Metabolic stimulation of mouse spleen lymphocytes by low doses of delta9-tetrahydrocannabinol. *Life Sci* **60**:1709–1717.

Scheer A and Cotecchia S (1997) Constitutively active G protein-coupled receptors: potential mechanisms of receptor activation. *J Recept Signal Transd Res* **17**:57–73.

Shire D, Calandra B, Rinaldi-Carmona M, Oustric D, Pessègue B. Bonnin-Cabanne O, Le Fur G, Caput D and Ferrara P (1996) Molecular cloning expression and function of the murine CB2 peripheral cannabinoid receptor. *Biochim Biophys Acta* **1307**:132–136.

Slipetz DM, O'Neill GP, Favreau L, Dufresne C, Gallant M, Gareau Y, Guay D, Labelle M and Metters KM (1995) Activation of the human peripheral cannabinoid receptor results in inhibition of adenylyl cyclase. *Mol Pharmacol* **48**:352–361.

Smit MJ, Leurs R, Alewijnse AE, Blauw J, Van Nieuw Amerongen GP, Van de Vrede Y, Roovers E and Timmerman H (1996) Inverse agonism of histamine H2 antagonists accounts for upregulation of spontaneously active histamine H2 receptors. *Proc Natl Acad Sci USA* **93**:6802–6807.

Stella N, Schweitzer P and Piomelli D (1997) A second endogenous cannabinoid that modulates long-term potentiation. *Nature* **388**:773–778.

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